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ANTIMALARIAL ACTIVITY OF CRUDE EXTRACT AND SOLVENT FRACTIONS OF THE STEM BARK OF *Periploca linearifolia* QUART.-DILL. & A.RICH. *(*ASCLEPIADACEAE) AGAINST *PLASMODIUM BERGHEI* IN MICE.

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This is to certify that the thesis prepared by Wubetu Yihunie, entitled: Anti-malarial activity of crude extract and solvent fractions of *Periploca linearifolia* Quart.-Dill. & A.Rich. stem bark in *Plasmodium berghei* infected mice and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin based Combination Therapy
CDC	Centers for Disease Control and Prevention
CelTOS	Cell-Traversal protein for Ookinetes and Sporozoites
DHFR	Dihydro Folate Reductase
DHPS	Dihydro Pteroate Synthase
MST	Mean Survival Time
OECD	Organization for Economic Cooperation and Development
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PfPI3K	*P. falciparum* Phosphatidyl Inositol-3-Kinase
SPSS	Statistical Packages for Social Science
TBVs	Transmission Blocking Vaccines
TM	Traditional Medicine
WHO	World Health Organization

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ABSTRACT

Background: The emergence of drug resistance to *P. falciparum* becomes challenging. Thus, a continued search for other effective, safe and cheap plant-based antimalarial agents becomes imperative in the face of these difficulties.

Objective: This study was aimed to investigate the antimalarial potentials of the crude stem bark extract and solvent fractions of *P. linearifolia* in *Plasmodium berghei* infected mice.

Methods: The dried stem bark of *P. linearifolia* was pulverized and extracted with 80% methanol. The dried crude extract was then further fractionated with n-hexane, chloroform, and ethyl acetate. For suppressive test, after three hours post inoculation of *P. berghei*, the first group was administered the vehicle 10 ml/kg. The second group was given chloroquine 25 mg/kg. Whereas the remaining three groups were administered 200, 400 and 600 mg/kg of the crude extract and 100,200 and 400 mg/kg of each of the chloroform, ethyl acetate and aqueous fractions. Furthermore, the crude extract and the aqueous fraction were evaluated using Rane's model for the curative test. The result was statistically analyzed using paired T-test and one-way analysis of variance (ANOVA). The results were considered significant at $P < 0.05$.

Result: In the suppressive test, the crude extract and fractions suppressed parasitemia level significantly ($P<0.05$ for 200 and 400 mg/kg, $P<0.001$ for 600 mg/kg of the crude extract; $P<0.01$ for 100 mg/kg, $P<0.001$ for 200 and 400 mg/kg of the aqueous and $P<0.01$ for 200 mg/kg, $P<0.001$ for 400 mg/kg of chloroform fractions). Whereas, in curative model 400 mg/kg of the aqueous fraction ($P<0.01$) and 600 mg/kg of the crude extract ($P<0.001$) showed a significant parasitemia level reduction. Both 400 and 600 mg/kg of the crude extract and 400 mg/kg of aqueous and chloroform fractions reversed reduction in packed cell volume.

Conclusion: The results indicated that the plant is nontoxic at 2gm/kg and has a promising antiplasmodial activity against *Plasmodium berghei*, which upholds the earlier *in vitro* findings. Thus, it could be considered as a potential source to develop new antimalarial agents.

Key Words: Antimalarial, *Plasmodium berghei*, *Periploca linearifolia*, parasitemia.

1. INTRODUCTION

1.1. Overview of Malaria

Malaria is one of the most common infectious diseases worldwide, particularly in Africa and south Asia. The number of malaria cases globally is about 212 million. More than 90% of the cases have occurred in the WHO African Region, followed by the WHO South-East Asia Region and the WHO Eastern Mediterranean Region. Globally, *Plasmodium vivax* accounts about 4% of estimated cases, but outside the African continent its infection proportion is 41% (1).

On the other hand, the number of malaria deaths globally is around 429 000 of which 70% are children. More than 92% deaths were in the WHO African Region followed by the WHO South-East Asia Region and the WHO Eastern Mediterranean Region. *Plasmodium falciparum* malaria claimed the vast majority of deaths (99%) followed by *P. vivax* which was responsible for 3100 deaths, with 86% occurring outside Africa (1). Ignorance of the epidemiological diversity that characterizes Africa and the challenges it poses to sustained control and elimination will be the single largest threat to the global eradication agenda (2).

Global financing for malaria control estimated to be US$ 2.9 billion in 2015. Of the total invested in the same year, international investments accounted for 68% and governments of malaria endemic countries for 32% (1). In Africa today, malaria becomes both a disease of poverty and a cause of poverty. Economists believe that malaria is responsible for a growth penalty of up to 1.3% per year in some African countries (3).

1.2. Life Cycle of Plasmodium

The natural ecology of malaria involves malaria parasites infecting successively humans and female *Anopheles* mosquitoes. In humans, the parasites grow and multiply first in the liver cells and then in the red cells of the blood. In the blood parasites grow inside the red cells and destroy them, releasing merozoites that continue the cycle by invading other red blood cells. The blood stage parasites are those that cause the symptoms of malaria. When gametocytes are picked up by a female *Anopheles* mosquito during a blood meal, they start another cycle of growth and multiplication in the mosquito (4).

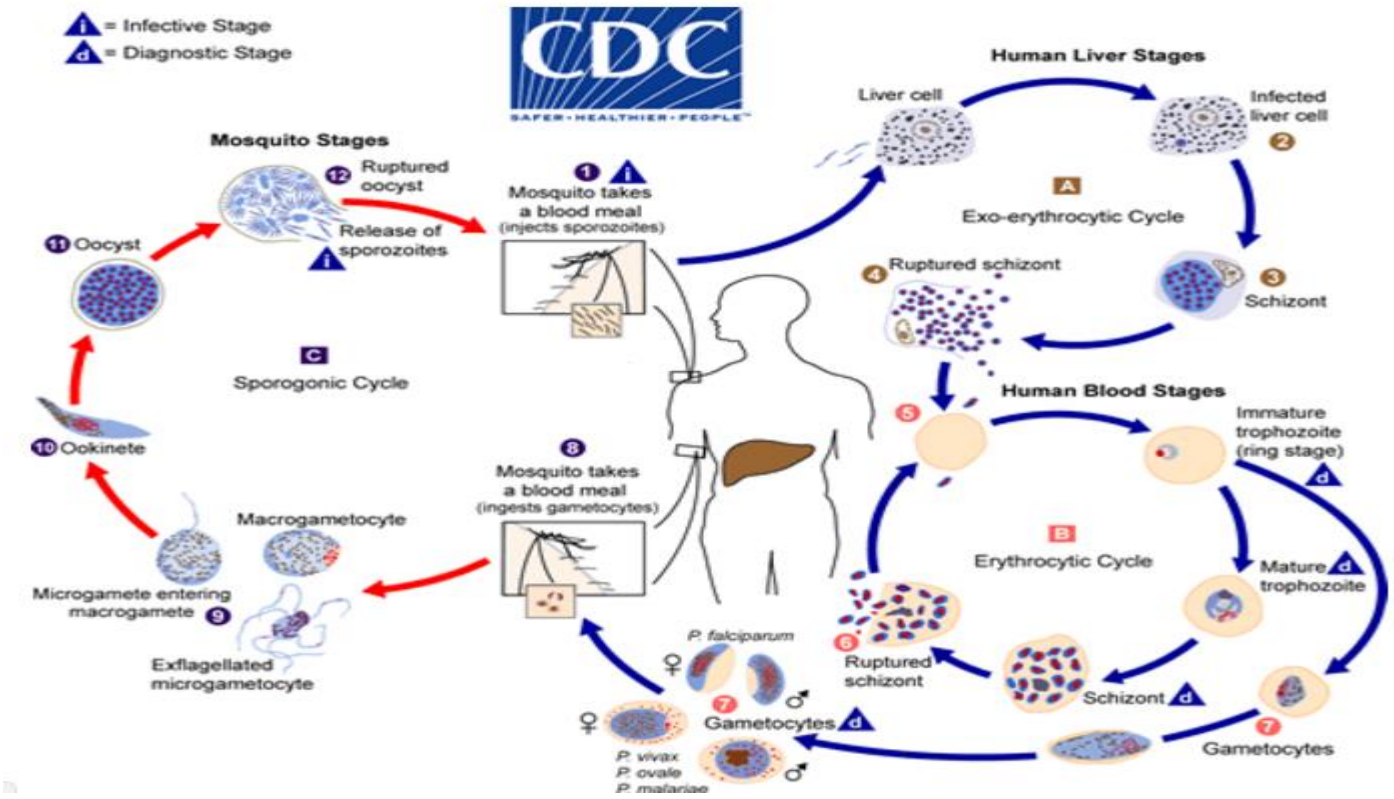

Figure 1. Life cycle of the malaria parasite (4).

During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host[1]. Sporozoites infect liver cells[2] and mature into schizonts[3], which rupture and release merozoites[4]. After this initial replication in the liver[A], the parasites undergo asexual multiplication in the erythrocytes[B]. Merozoites infect red blood cells[5]. Some parasites differentiate into sexual erythrocytic stages (gametocytes)[7]. The gametocytes are ingested by an *Anopheles* mosquito during a blood meal[8]. The parasites' multiplication in the mosquito is known as the sporogonic cycle[C]. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes[9]. The zygotes in turn become motile and elongated (ookinetes)[10] which invade the midgut wall of the mosquito where they develop into oocysts[11]. The oocysts grow, rupture, and release sporozoites[12], which make their way to the mosquito's salivary glands. Inoculation of the sporozoites[1] into a new human host perpetuates the malaria life cycle (4).

1.3. Etiology and symptoms of malaria of Malaria

Malaria in humans is caused by five species of parasites belonging to the genus *Plasmodium*. These include *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (5, 6). Even though malaria parasites are commonly detected in blood donors, transfusion transmitted malaria occurs infrequently (7).

Symptoms of malaria are generally non-specific and most commonly consist of fever, malaise, weakness, nausea, vomiting, diarrhea, dizziness, confusion, disorientation, coma, headache, back pain, myalgia, chills and cough. On the other hand, malaria can be diagnosed with microscope, rapid diagnostic tests and polymerase chain reaction (PCR) (6, 8).

1.4. Overview of Treatment of Malaria

1.4.1. Antimalarial Drugs and Their Targets

1.4.1.1. Cytosolic Targets

Folate metabolism pathway is one of the drug targets in the cytosol. Unfortunately, resistance to individual dihydro folate reductase (DHFR) and dihydro pteroate synthase (DHPS) inhibitors, including pyrimethamine, proguanil and sulfas, leads to a marked loss in efficacy of even combination regimens (9). Similarly, 5-fluoroorotate exerts antimalarial activity via the inhibition of thymidylate synthase (10).

Malaria parasites are dependent on glycolysis for energy production. *P. falciparum* lactate dehydrogenase's unique binding site for the NADH cofactor offers opportunities for the design of selective inhibitors (11). Malaria parasites cannot synthesize purines and rely on salvage of host purines for nucleic acid synthesis. The principal source of purines in *P. falciparum* appears to be hypoxanthine, and hypoxanthine–guanine phosphoribosyltransferase (HGPRT) has been considered as a potential drug target (12).

1.4.1.2. Parasite Membrane Targets

The most abundant lipid in *Plasmodial* membranes is phosphatidylcholine. Synthesis of phosphatidylcholine requires host choline, and blockage of choline transport has been identified as a promising therapeutic strategy (13). A lead compound, G25 inhibited the development of cultured *P. falciparum* (14, 15).

1.4.1.3. Food Vacuole Targets

Antimalarial drugs appear to act by preventing hemozoin formation, producing free radicals in the food vacuole or, in the case of experimental compounds, preventing globin hydrolysis (16). Cysteine and aspartic parasite proteases (falcipains and plasmepsins, respectively) involved in the degradation of haemoglobin are also potential targets (17).

The 4-aminoquinoline chloroquine appears to act by blocking the formation of hemozoin from heme molecules once they are liberated from hemoglobin (18). The food vacuole also appears to be the target of artemisinin antimalarials. Artemisinins contain an endoperoxide bridge that is essential for antimalarial

activity and that appears to undergo an iron-catalyzed decomposition into free radicals (19). The compounds apparently exert antimalarial effects via free radical damage, possibly by alkylation of *Plasmodial* proteins (20, 21).

1.4.1.4. Mitochondrial Targets

One antimalarial drug has a mitochondrial target. Atovaquone acts against ubiquinol–cytochrome *c* oxidoreductase (complex III), inhibits electron transport and collapses mitochondrial membrane potential, which is required for a number of parasite biochemical processes (22).

1.4.1.5. Apicoplast Targets

The apicoplast maintains certain specific functions, probably including fatty acid, heme and amino acid metabolism. Like the mitochondrion, the apicoplast has a separate, prokaryote-like genome, and this fact probably explains the antimalarial effects of a number of antibacterial compounds that otherwise do not attack eukaryotes (23). Many compounds probably act by targeting apicoplast and/or mitochondrial processes that are similar to those in bacteria (24). Tetracycline, clindamycin, macrolides and chloramphenicol inhibit different steps of prokaryote-like protein synthesis. Quinolone antibiotics inhibit DNA gyrase, and rifampin inhibits RNA polymerase, again with specificity to prokaryote-like activity (25).

1.4.2. Treatment of Malaria

The WHO recommended package of core interventions to prevent infection and reduce morbidity and mortality comprises vector control, chemoprevention, diagnostic testing and treatment (26).

1.4.2.1. Uncomplicated Malaria

***P. falciparum* or species not identified (acquired in areas without chloroquine resistance)**

For *P. falciparum* infections acquired in areas without chloroquine-resistant strains patients can be treated with oral chloroquine (27, 28).

***P. falciparum* or species not identified (acquired in areas with chloroquine resistance)**

For *P. falciparum* infections acquired in areas with chloroquine resistance, four treatment options are available. The first two treatment options are atovaquone-proguanil or artemether-lumefantrine, which can be used for adult and pediatric patients. Quinine sulfate plus doxycycline, tetracycline, or clindamycin is the next treatment option. The fourth option, mefloquine, is associated with rare but potentially severe neuropsychiatric reactions when used at treatment doses (27, 28).

P. malariae* and *P. knowlesi

For both of these infections chloroquine may still be used. In addition, any of the regimens listed above for the treatment of chloroquine-resistant malaria may be used for the treatment of *P. malariae* and *P. knowlesi* infections (27, 28).

P. vivax* and *P. ovale

Chloroquine remains an effective choice for all *P. vivax* and *P. ovale* infections except for *P. vivax* infections acquired in Papua New Guinea or Indonesia. The regimens listed for the treatment of *P. falciparum* are also effective and may be used. The three treatment regimens for chloroquine-resistant *P. vivax* infections are quinine sulfate plus doxycycline or tetracycline, or, Atovaquone-proguanil, or mefloquine (27, 28).

In addition to requiring blood stage treatment, infections with *P. vivax* and *P. ovale* can relapse due to hypnozoites that remain dormant in the liver. To eradicate the hypnozoites, patients should be treated with a 14-day course of primaquine phosphate. For pediatric patients, the treatment options are the same as for adults except the drug dose is adjusted by patient weight (27, 28).

Alternatives for Pregnant Women

Malaria infection during pregnancy can lead to miscarriage, premature delivery, low birth weight, congenital infection and perinatal death. For pregnant women diagnosed with uncomplicated malaria caused by *P. malariae, P. vivax, P. ovale,* or chloroquine-sensitive *P. falciparum* infection, chloroquine is recommended. For pregnant women diagnosed with uncomplicated malaria caused by chloroquine-resistant *P. falciparum* infection, either mefloquine or a combination of quinine sulfate and clindamycin is recommended. For pregnant women diagnosed with uncomplicated malaria caused by chloroquine-resistant *P. vivax* infection, mefloquine is recommended (27-29).

For pregnant women diagnosed with uncomplicated malaria caused by chloroquine-resistant *P. falciparum* infection, atovaquone-proguanil or artemether-lumefantrine may be used if other treatment options are not available or are not being tolerated, and if the potential benefit is judged to outweigh the potential risks (28).

1.4.2.2. Severe Malaria

Patients who are considered to have manifestations of more severe disease should be treated aggressively by quinidine gluconate or parenteral artesunate. Once the parasite density is < 1% and the patient can take oral medication, the patient can complete the treatment course with an oral regimen such as oral quinine at the same dosage for uncomplicated malaria for a combined treatment course of quinidine/quinine. Other oral

regimens such as atovaquone-proguanil or artemether-lumefantrine may be used instead of an oral quinine based regimen (27, 28).

1.5. Antimalarial Drug Resistance and Vector Resistance

Currently, mosquitos become resistant to insecticides which jeopardizes malaria control. Therefore, failure to address the issue of insecticide resistance could undermine regional efforts to contain and eliminate artemisinin resistant parasites. The effectiveness of these interventions depends on the effectiveness of the insecticides used. Pyrethroids are currently the only insecticides available for treatment of mosquito nets (30).

Antimalarial drug resistance is mediated by two processes. These are the rate that de novo mutations conferring resistance appear and are selected through drug use within an individual and the spread of those resistant alleles to other individuals. High mutation rates at the cellular level, which provide a means of continually evading the immune system, offer a mechanism for selection of resistance within a host, while interactions between other parasites and their hosts due to variation in transmission and host susceptibility and influence the probability of selection at the population level (31, 32).

1.5.1. Drug resistance genetic markers

Identification of potent drug-resistant molecular marker is an important tool to determine the emergence and spread of antimalarial drug resistance worldwide. Genetic crosslink and mapping studies of parasites assisted in identifying the genetic markers for drug resistance (33). Some of the genetic markers involved in drug resistance are listed below.

1.5.1.1. *P. falciparum* Chloroquine Resistance Transporter (PfCRT)

PfCRT protein belongs to the drug/metabolite transporter superfamily and chloroquine resistance transporter-like transporter family with 10 putative transmembrane domain spanning the digestive vacuole membrane of the parasite (34). The K76T mutation in Pfcrt gene is the primary determinant of chloroquine resistance and susceptibility (35). Variation in the PfCRT protein influences antimalarial drug susceptibility and resistance to quinine, amodiaquine, piperaquine, and lumefantrine (36-39).

1.5.1.2. *P. falciparum* Multidrug Resistance Protein 1 (PfMDR1)

PfMDR1 protein is a transmembrane protein present in the digestive vacuole of the parasite similar to PfCRT protein and belongs to the ATP-binding cassette (ABC) superfamily (40, 41). Mutation in Pfmdr1 gene at the following position (N86Y, Y184F, S1034C, N1042D, and D1246Y) have been reported to involve in determining the drug susceptibility to chloroquine, quinine, mefloquine, halofantrine, lumefantrine, and

artemisinin (42-45). In addition, copy number variation of Pfmdr1 gene has been linked to higher level of resistance to quinine, mefloquine, halofantrine, lumefantrine, and artemisinin (46).

1.5.1.3. *P. falciparum* Multidrug Resistance-associated Protein (MRP)

MRP helps in the transport of organic anionic substrates such as oxidized glutathione, glucuronate, sulfate conjugates, and also in drug transport (47). Two mutations at position Y191H and A437S in PfMRP were found associated with CQ and quinine resistance (48). Thus, PfMRP is involved in varying the antimalarial response to resistance but not in determining the drug resistance mainly, and also hypothesized that PfMRP protein effluxes various metabolites and drugs out of the parasite in association with other transporters (49).

1.5.1.4. Cytochrome B

Cytochrome b (cytb) gene is a subunit of cytochrome bc1 complex, which catalyses the transfer of electrons across the inner mitochondrial membrane to maintain the electrochemical potential of the membrane (50). The antimalarial drug atovaquone binds to the ubiquinol binding site of cytb, thus disrupting the electrochemical potential of the mitochondrial membrane, which is fatal for the parasite survival (50). Single mutation at Y268N/S/C codon in the cytb gene associated with resistance to atovaquone in *P. falciparum* field isolates (42).

1.5.1.5. Kelch 13 (K13 protein)

The biochemical and cellular evidence showed that artemisinins are potent inhibitors of *P. falciparum*phosphatidylinositol-3-kinase (PfPI3K). In resistant clinical strains, increased PfPI3K was associated with the C580Y mutation in *P. falciparum* Kelch13 (PfKelch13), a primary marker of artemisinin resistance. The C-terminal region of K13 protein has six kelch motifs consisting of beta sheets that folded into a propeller domain and mutation in this region is predicted to disrupt the domain scaffold and alter its function. The kelch family proteins have diverse cellular functions, such as in organizing and interacting with other proteins (51, 52). Polyubiquitination of PfPI3K and its binding to PfKelch13 were reduced by the PfKelch13 mutation, which limited proteolysis of PfPI3K and thus increased levels of the kinase, as well as its lipid product phosphatidylinositol-3-phosphate (PI3P) (53, 54).

1.6. Antimalarial Vaccine Development

An ideal malaria vaccine requires three essential features: (i) multiple components that will induce an effective immune response to the different stages of the malaria infection; (ii) multiple epitopes that are restricted to presentation by different major histocompatibility complex (MHC) molecules; and (iii)

multiimmunogenicity inducing more than one type of immune response, including cell-mediated and humoral components (55-57).

1.6.1. Transmission-Blocking Vaccines (TBVs)

Transmission-blocking vaccines target antigens on gametes, zygotes and ookinetes to prevent parasite development in the mosquito midgut. The aim of these vaccines is to induce antibodies against the sexual-stage antigens to block ookinete to oocyst transition to stop the subsequent generation of infectious sporozoites (58). One of the leading vaccine candidates in this group includes the *P. falciparum* ookinete surface antigens Pfs25 (59).

1.6.2. Pre-erythrocytic vaccines

The liver stage of *P. falciparum* is an attractive therapeutic target for the development of both antimalarial drugs and vaccines, as it provides an opportunity to interrupt the life cycle of the parasite at a critical early stage (60). To date, the most advanced pre-erythrocytic vaccine candidate is RTS,S/AS01, which consists of a truncated circumsporozoite protein (CSp) of *P. falciparum* directly fused to the hepatitis B surface antigen. A Phase 3 trial of RTS,S/ASO1 began in May 2009 and ended in early 2014, with 15 459 children in seven countries in sub-Saharan Africa (Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, and Tanzania) (61). The WHO Regional Office for Africa (WHO/AFRO) announced on April 24, 2017, that Ghana, Kenya, and Malawi will collaborate with WHO in the Malaria Vaccine Implementation Programme (MVIP) that will make the RTS,S/ASO1 vaccine available in these three countries, beginning in 2018 (62). A novel antigen, the cell-traversal protein for ookinetes and sporozoites (CelTOS), has been identified as an essential protein for the traversal of the malaria parasite in both mammalian and the insect hosts (63).

1.6.3. Blood-stage vaccines

From the several blood-stage antigens in clinical trials: apical membrane antigen 1 (AMA1) (64) and erythrocyte-binding antigen-175 (EBA-175) are some (65). The extensive genetic diversity of the parasite and the selective pressure exerted by the host's immune response are major factors to be considered in the development of effective blood-stage vaccines (66).

1.7. Herbal/Traditional Medicine as an Antimalarial Agent

Traditional medicine (TM) is the oldest form of health care in the world and is used in the prevention, and treatment of physical and mental illnesses (67, 68). Such forms of medicine as traditional Chinese medicine

(TCM), Ayurveda, Kampo, traditional Korean medicine (TKM), and Unani employ natural products and have been practiced all over the world for hundreds or even thousands of years (69, 70).

Through its use of natural products, TM plays a great role in the discovery of lead compounds and drug candidates. In TM, a single herb or formula may contain many phytochemical constituents, such as alkaloids, terpenoids, flavonoids, etc. Generally, these chemicals function alone or in conjunction with one another to produce the desired pharmacological effect (71, 72).

Cinchona specious was traditionally used as an antimalarial remedy by the Incas in Peru. Quinine, the first antimalarial drug, is considered the prototype for the development of synthetic 4- and 8- aminoquinoline classes of antimalarial drugs, as exemplified by chloroquine and primaquine, respectively (73).

The discovery of artemisinin can be traced back to the 1960s, when tropical malaria was a serious problem during the Vietnam War. North Vietnam requested China to help tackle the malaria problem. The Chinese government approved a project for malaria control and drug research in 1967. As part of the phytochemical and pharmacological research effort, a lot of Chinese herbal medicines were screened and investigated with respect to their toxicity or efficacy. Eventually artemisinin was derived from *Artemisia annua* L. in 1972 (74-76).

1.8. The experimental plant

1.8.1 The Family-Asclepiadaceae

Asclepiadaceae is a large family of 250 genera and over 2000 species: widespread in tropical and subtropical regions, especially in Africa and southern South America, with a moderate representation in northern and southeastern Asia. Members of this family are herbs, shrubs, or rarely treelike, with milky or, less often, clear latex (77, 78).

1.8.2. The sub family Periplocoideae (Apocynaceae)

It comprises 195 species belonging to 33 genera with most of the genera containing only a few species (79). Periploca was described by Linnaeus (1753) and is the type genus of the subfamily Periplocoideae (77, 79).

From the plant species of Apocynaceae family in the Brazillian Amazon, Andrade Neto *et al*. isolated antiplasmodial indole alkaloids (80). The methanolic extracts from *Carissa edulis* (Apocynaceae) root bark were moderately active against the chloroquine sensitive strain of *P. falciparum* (81).

Both stem and leaf parts of *Ochrosia akkeringae* and *Tabernaemontana pandacaqui* (Apocynaceae) contain alkaloids and have potential antimalarial activity (81). Stem bark of ethanolic extract and chloroform fraction of *Aspidosperma nitidum* (Apocynaceae) showed a promising antimalarial activity (82).

Aspidosperma olivaceum (Apocynaceae), which is used to treat fevers in some regions of Brazil, contains the monoterpenoid indole alkaloids aspidoscarpine, uleine, apparicine, and N-methyl-tetrahydrolivacine which are active *in vitro* against erythrocytic stages of *P. falciparum* (83).

Cryptolepis sanguinolenta Lindl. Schltr. (Periplocaceae) is used for the treatment of fevers. It is used for urinary tract infections, especially *Candida*, inflammatory conditions, malaria, hypertension, microbial infections and inflammatory conditions, stomach aches colic (84). Active principals identified are indole quinoline alkaloids (85).

1.8.2. The genus *Periploca*

Many chemical compounds have been isolated and identified from species of the *Periploca* genus, such as *α*- and *β*-amyrin, lupeol and *β*-sitosterol from *Periploca laevigata* (86). Moreover, several lupene-type triterpenes and elemane-type sesquiterpenes have been isolated from *Periploca aphylla* and roots of *P. laevigata* respectively (86, 87). Lupeol, *β*-amyrin and *β*-sitosterol (terpenoids) inhibited both chloroquine sensitive and resistant strains of *P. falciparum* (88).

1.8.3 *Periploca linearifolia* Quart.-Dill. & A.Rich. (Asclepiadaceae)

P. linearifolia (also known as “Moyder” in Amharic) is a liana, woody at base, up to 10 m long, glabrous and latex copious. Leaves are opposite, simple and entire, almost sessile; stipules are absent. Flowers are bisexual; pedicle is 5-10 mm long; sepals are ovate; whereas lobes are slightly fleshy and glandular, spreading oblong-ovate. Fruit comprises a pair of follicles, which are horizontal. Each follicle is linear-ovoid, which are many seeded. Seeds are ovate, brown, tuft of hairs white. The plant occurs in eastern Central Africa, East Africa and part of southern Africa, from Ethiopia south to eastern DR Congo, Malawi and Zimbabwe (89).

Figure 2. Morphological view of *Periploca linearifolia* Quart.-Dill. & A.Rich.

1.8.4. Reported Ethnobotanical Use and *in vitro* studies *P. linearifolia*

Hot water decoction of root bark of *P. linearfolia* is used as antimalarial in Meru district, Kenya (90). Leaves are macerated in water and used as a dressing to treat mastitis. A leaf decoction is drunk to treat diarrhea, intestinal worms and insanity. The leaf sap in water or a root decoction is taken by pregnant women as a tonic and to stop vomiting. Pounded leaves in water are taken to treat female sterility (89).

A root bark infusion or decoction is drunk or applied as enema to treat tapeworm and other intestinal worms. Roots cooked in soup are eaten to treat fever, malaria, chest complaints, including cough and pneumonia, female infertility and venereal diseases. Root powder and latex is externally applied to hemorrhoids. The leaves and fruits of *P. linearifolia* are used for the eradication of lice in goats and sheep (89, 91).

An *in vitro* antimalarial test with a methanolic stem bark extract of *P. linearifolia* showed highly significant antiplasmodial activity *in vitro* against a chloroquine sensitive and resistant strain of *P. falciparum.* In addition, a chloroform stem bark extract showed a moderate antiplasmodial activity *in vitro* against a chloroquine sensitive strain. The extracts showed low toxicity in a brine shrimp cytotoxicity assay. A methanolic extract of the leafy stem showed significant antibacterial activity and moderate antifungal activity *in vitro* (88, 89, 92).

Three compounds namely Lupeol, β- sitosterol and β- amyrin which were isolated from the root bark of *P. linearifolia* found to inhibit both chloroquine sensitive and resistant strains of *P. falciparum* (88).

1.8.5. Phytochemical constituents of *P. linearifolia* Quart.-Dill. & A.Rich.

The leaf extracts from *P. linearifolia* contain phytin, anthraquinone, gemnamagenin, gymnemic acid II, betaine, cholin and lupeol (93, 94). Three terpenoids namely, Lupeol, β- sitosterol and β- amyrin were isolated from the root bark of *P. linearifolia* (88).

1.9. Justification of the Study

Due to the pandemic nature and fatality of malaria and the emergence of antimalarial drug resistance, especially to chloroquine and artemisinin derivatives, there is a high interest of developing new and affordable antimalarial drugs. To mention some of the challenges, the most important one is *Plasmodium* parasites are resistant to the most widely available, affordable and safest antimalarial drugs. Moreover, currently available indoor spraying with insecticides to reduce the transmission of malaria is hampered by insecticide resistance (1, 95). Indeed, artemisinin resistance to a deadly *P. falciparum* now poses a threat to the control and elimination of malaria. Chloroquine resistant strains can be controlled by artemisinin and artemisinin derivatives. But, currently there is no alternative approved antimalarial drug to replace artemisinin derivatives. Hence, it is extremely important to urgently intensify research in the development of new, cheap and effective antimalarial drugs. Medicinal plants remain the main focus by scientists as a noble source of new lead compounds in the development of new antimalarial agents.

Hence, Ethiopia, with a wealth of natural resources, is an ideal place to search for new drugs from plants. The finding of this experimental study helps the scientific community to further investigate on the plant, *P. linearifolia*, by initiating advanced studies on formulation and molecular mechanisms of plant source drugs and by identifying and isolating a specific antimalarial compound. Unless such types of screening studies are carried out and potential new antimalarial drugs discovered, the drug resistance phenomenon may lead us to the pre-antibiotic/antimicrobial era. Ethiopia being rich in flora which can be sources of different new antimalarial agents, further screening of plants is important.

An *in vitro* antimalarial test with a methanolic and chloroform extract of the stem bark of *P. linearifolia* conducted in Kenya, showed a promising antiplasmodial activity against a chloroquine sensitive and resistant strains. This plant is used for the treatment of malaria in the Lake Victoria region and Meru district, Kenya (92). Therefore, the present study describes the *in-vivo* antimalarial activity of crude extract and solvent fraction of *P. linearifolia* in mice infected with chloroquine sensitive *P. berghei.*

2- OBJECTIVES

2.1. General Objective

- ✓ To evaluate the antimalarial activity of crude extract and solvent fractions of the stem bark of *Periploca linearifolia* Quart.-Dill. & A.Rich. (Asclepiadaceae) against *Plasmodium berghei* in mice.

2.2. Specific Objectives

- ✓ To carry out preliminary phytochemical screening of the crude hydroalcoholic extract of *P. linearifolia;*
- ✓ To determine the acute oral toxicity of the hydroalcoholic extract of *P. linearifolia;*
- ✓ To evaluate the *in vivo* antimalarial activity of the hydroalcoholic extract of *P. linearifolia;*
- ✓ To evaluate the *in vivo* antimalarial activity of the solvent fractions

3. METHODS

3.1. Drugs and Chemicals

Normal isotonic saline (Euro-med Laboratories, Phillippines), absolute methanol (ReAgent chemical services, UK), n-hexane, 2% tween 80, hydrochloric acid (Blulux laboratories, India), ethyl acetate (Loba chemicals, India), chloroform (Atico, India), citrate dextrose (Deluxie Scientific Surgico, India), giemsa (Science Lab, USA), ethanol (Nice chemicals, India), ferric chloride (Fisher Scientific CO, USA), sodium hydroxide sulfuric acid (Supertech, India) and Mayer's reagent (Avishkar Lab Tech Chemicals, India) were used.

3.2. Materials

Electrical balance, 1 ml syringes (Hindustan, India), needles Shandong Zibo, China), vials, examination glove, permanent marker, Whatman filters paper No.1 (Whatman ®, England) , collecting flask, separatory fennel, hematocrit centrifuge, Micro-Hematocrit Reader (Hettich, Germany), microscope (Olympus, Japan) and laboratory glass wares were used.

3.3. Plant Material

Stem bark of *P. linearifolia* plant was collected from Lay Armachiho Woreda, near Tikil Dingay town, North Gondar Zone, 23 kms North of Gondar city on December, 2016. The collected plant material was wrapped with plastic sheets during transportation. The plant was identified as *Periploca linearifolia* Quart.-Dill. & A.Rich. by Mr. Abiyu Enyew (botanist) and a voucher specimen was deposited (No. WY001) at the Herbarium of biology department, University of Gondar for future reference.

3.4. Experimental Animals

Healthy, adult Swiss albino mice of either sex (22-32 g, and 6–8 weeks of age) were obtained from the animal house of the School of Pharmacy, College of Medicine and Health Science (CMHS), University of Gondar (UoG). The animals were housed in cages under standard conditions and provided with pellet diet and water *ad libitum*. The animals were allowed to acclimatize to the laboratory condition for a week before beginning the experiment. All protocols were performed based on the international animal care and welfare guidelines (96). The study protocol was approved by the ethical review board of Department of Pharmacology, School of Pharmacy, UoG. The experiment was conducted following approval by the above body for the use of animals for experiment.

3.5. Parasite

Chloroquine sensitive strain of *P. berghei* (ANKA strain) obtained from the Ethiopian Public Health Institute, Addis Ababa was used. The parasites were maintained by serial passage of blood from infected mice to non-infected ones on weekly basis.

3.6. Extract preparation

The stem bark was peeled from the plant, air dried at room temperature under shade and reduced to appropriate size. A total of 1.2 kg dried stem bark was extracted by maceration (100 g of dried stem bark in 600 ml of 80% methanol) for 72 h. The mixture was first filtered using gauze and then with Whatman filters paper No. 1 (Whatman ®, England). The residue was re-macerated for another 72 hour twice and filtered. The combined filtrates were then put under oven at a temperature not more than 40°C. After evaporating methanol and some part of the aqueous part, it was transferred to a desiccator for further drying. The dried extract was kept in a vial and stored in a refrigerator at −4°C until use (97).

3.7. Preparation of fraction

The crude 80% methanolic extract was dissolved in distilled water and successively partitioned using different solvents of increasing polarity (n-hexane, chloroform, and ethyl acetate) in a separatory funnel as shown schematically in Figure 3. The different solvent fractions were concentrated and dried in oven under 40 ^{0}C. The aqueous fraction was dried by lyophilizer. The dried fractions were then transferred into separate vials and stored in a refrigerator at −4°C for further use.

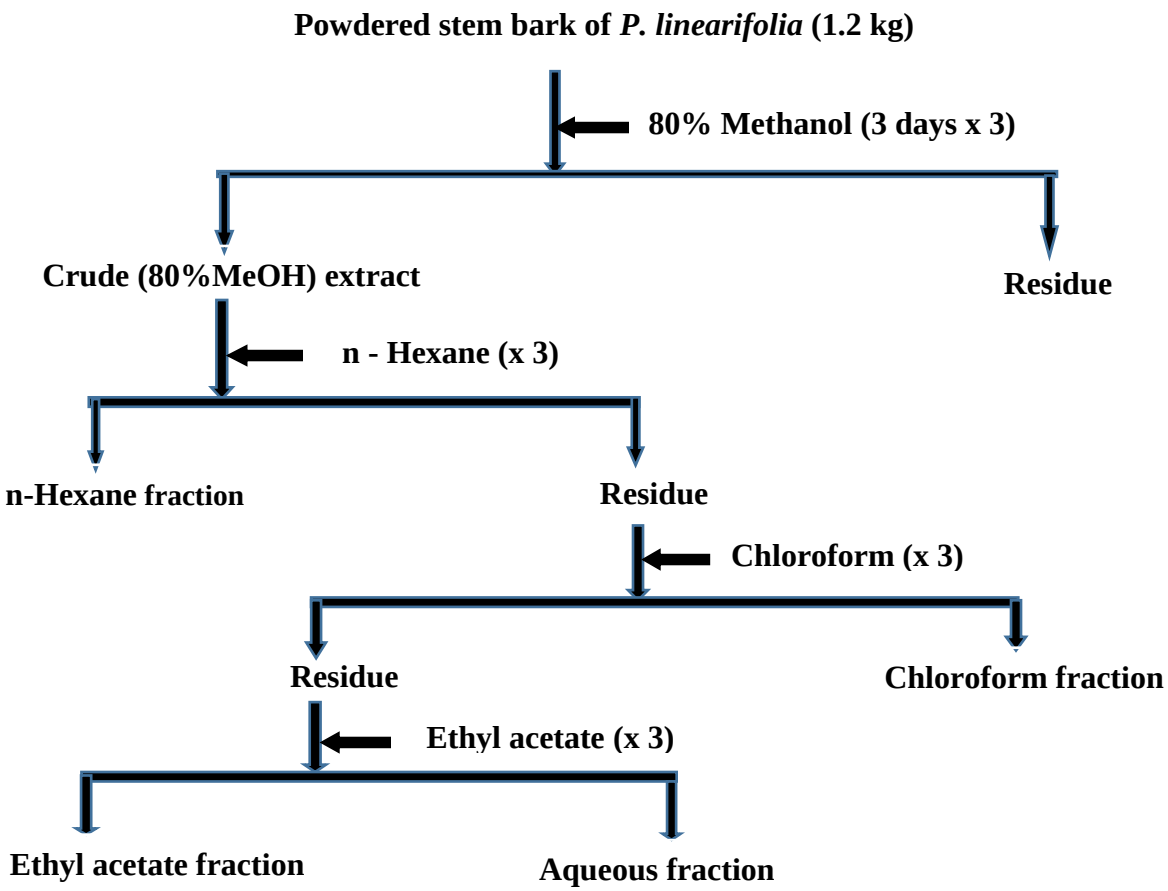

Figure 3. Schematic diagram for extraction and fractionation of the stem bark of *P. linearifolia*

3.8. Preliminary Phytochemical Screening

The 80% methanolic extract of *P. linearifolia* stem bark was screened for the presence of secondary metabolites to relate the antimalarial activity of the plant with the presence or absence of these constituents. Thus the tests for alkaloids, saponins, flavonoids, terpenoids, phenols and tannins were performed according to standard tests as described below.

Test for Alkaloids

Crude extract was mixed with 2 ml of 1% hydrochloric acid (HCl) and heated gently. Mayer's and/or Wagner's reagents were then added to the mixture. Presence of alkaloid was confirmed by turbidity of the resulting precipitate (98).

Tannins test (Braymer's Test)

2 ml of extract was mixed and then treated with 2-3 drops of ferric chloride (5%) and formation of green precipitate was used as indication of presence of tannins (99).

Test for terpenoids

Crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated sulfuric acid (H_2SO_4) was added and heated for about 2 minutes. Formation of grayish color indicated the presence of terpenoids (99).

Test for flavonoids (Alkaline reagent test)

Crude extract was mixed with 2 ml of 2% solution of sodium hydroxide solution. The formation of intense yellow color which becomes colorless upon the addition of a few drops of diluted H_2SO_4 showed the presence of flavonoids (99).

Test for saponins (foam test)

Crude extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously and stable foam formation was taken as an indication for the presence of saponins (98).

Test for phenols (Ferric chloride test)

A fraction of the extract was treated with aqueous 5% ferric chloride and was observed for formation of deep blue or black color which indicated presence of phenols (100).

3.9. Acute Oral Toxicity Study

Acute toxicity testing was conducted for the 80% methanol extract (single dose 2 g/kg) using Organization for Economic Cooperation and Development guidelines. The extract was evaluated for its toxicity in non-infected female Swiss albino mice aged of 6-8 weeks and weighing 26-32 g. The mice were fasted for three hours and weighted before test. A single female mouse was given 2 g/kg of the extract as a single dose by oral gavage. After administration of the extracts food was withheld for further two hours period. The mice was strictly followed for four hours and then for 24 hours. Since, death was not recorded in the first 24 hrs, another 4 female mice were given the same dose based on the outcome of the first animal. The animals were observed for toxicities like diarrhea, weight loss, absence of tremor, lethargy and paralysis periodically for the first four hours during the 24 h period and later were followed for 14 days for any adverse effect and lethality (96).

3.10. *In vivo* Antimalarial Activity Testing

P. berghei (ANKA strain) was used for induction of malaria in experimental mice. The parasites were maintained by intraperitoneal serial passage of blood. The infected blood was then collected in test tubes by decapitation and diluted with isotonic saline before it was given to the different groups of mice.

3.10.1. Suppressive test

3.10.1.1. Antimalarial activity test of the crude extract

The standard four-day suppressive test method was used (97, 101). Blood was taken from a donor mouse with approximately 20%-30% parasitemia and diluted in physiological saline to 5 x 10^7 parasitized erythrocytes per ml. Swiss albino mice weighing 22-32 g were infected with 0.2 ml (1 x 10^7 parasitized erythrocytes) *P. berghei* intraperitoneally (i.p.) and were randomly divided into five groups of five mice with three test groups and two control groups (each for chloroquine as a standard drug and distilled water as a negative control) (97).

The test extract was prepared in three different doses (200, 400 and 600 mg/kg) and chloroquine at 25 mg/kg. Each vehicle, extract or chloroquine were administered as a single dose daily. The extracts and the drug were given through oral route by using standard oral gavage. The dose levels of the extracts and fractions were selected for mice based on the result obtained from the oral acute toxicity test.

Treatment was started 3 hour post infection on day 0 and continued daily for four days (i.e. from day 0 to day 3). On the fifth day (D4) thin smears of blood films were obtained from the peripheral blood on the tail from each mouse. The smears were placed on microscopic slides which were fixed with methanol and stained with 10% giemsa at pH 7.2 for 15 min. The parasitemia level was determined by counting the number of parasitized erythrocytes out of four random fields of the microscope. Average percent parasitaemia and suppression was calculated by using the following formula (97, 101).

$$\%\ \text{Parasitemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC}} \times 100$$

$$\%\ \text{suppression} = \frac{\text{mean parasitemia of negative control} - \text{mean parasitemia of treated group}}{\text{mean parasitemia of negative control}} \times 100$$

3.10.1.2. Antimalarial activity test of fractions

Chloroform, ethyl acetate and aqueous fractions of the crude extract were administered with three different doses for each 100, 200 and 400 mg/kg orally daily to respective groups of five mice each 3 h post-infection intraperitoneally with 0.2 ml of infected blood containing about 1 x 10^7 *P. berghei*. A negative control group

was administered the 2% Tween 80 while chloroquine (25 mg/kg/day) was given to the positive control group. The administration was continued for four days. On the fifth day, thin films were prepared from tail blood of each mouse and the parasitemia levels were determined by counting the number of parasitized erythrocytes in four random fields of the microscope. The average percentage chemosuppression was calculated as shown under section 3.2.5.1.1 (97, 101).

3.10.2. Rane's Test

Evaluation of the curative potential of the crude extract and the aqueous fraction (the most active fraction in the four day suppressive test) were carried out according to the method described by Ryley and Peters (102). On Day 0, standard inocula of 1×10^7 infected erythrocytes were inoculated in mice intraperitoneally. Seventy-two hours later, mice were randomly divided into eight groups of five mice. The first three groups belonged to 200, 400 and 600 mg/kg of the crude extract and the other three groups were assigned for 100, 200 and 400 mg/kg of the aqueous fraction. Whereas the remaining two groups belonged for the vehicle distilled water 10 ml/kg and chloroquine 25 mg/kg.

All mice were dosed, as described in the four day suppressive test, orally once daily for four days. Geimsa stained thin blood film were prepared from the tail of each mouse daily for 5 days from day three to day seven to monitor parasitemia level. The average percentage parasitemia was calculated as shown under section 3.2.5.1.1. Mean survival time (MST) for each group was determined arithmetically by calculating the average survival time (days) of mice starting from date of infection over a period of 30 days (D0-D29).

$$\text{MST} = \frac{\text{Sum of survival time of all mice in group (days)}}{\text{Total number of mice in that group}}$$

3.10.3. Packed Cell Volume Measurement

Packed cell volume (PCV) was measured to predict the effectiveness of the test extract and solvent fractions in preventing hemolysis resulting from increasing parasitemia associated with malaria. Heparinized capillary tubes were used for collection of blood from tail of each mouse. The capillary tubes were filled with blood up to ¾th of their volume and were sealed at the dry end with sealing clay. The tubes were then placed in a micro-hematocrit centrifuge, with the sealed end outwards and was centrifuged for 5 min at 11,000 rpm. The tubes were then taken out of the centrifuge and PCV was determined using a standard Micro-Hematocrit Reader. PCV is a measure of the proportion of RBCs to plasma and measured before inoculating the parasite and at day four using the following relationship:

$$\text{PCV} = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}} \text{ x } 100\%$$

3.10.4. Monitoring of Body Weight and Temperature Changes

For a four day suppressive test and Rane's test, the body weights of the mice were determined to observe whether the stem bark extract or solvent fractions prevented weight loss. Weights were taken on day zero (D0) and day five (D4). Rectal temperature was measured by a digital thermometer before infection, four hours after infection and then daily. For Rane's test, body weight and temperature were measured before infection and from day 3–7 after infection.

3.10.5. Statistical Analysis

The raw datas obtained from the experiments were expressed as mean ± SEM (standard error of the mean). Statistical analyses were carried out by using SPSS Statistical software version 20. All the parametrs parasitemia level, % suppresson, mean survival time, PCV, temperature and weight were statistically analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's Multiple Comparison Test. PCV, temperature and weight were statistically analyzed using paired T- test to analyze the difference within groups before and after treatment. The results were considered significantly different when $p < 0.05$.

4. RESULT

4.1. Percentage yields of crude extract and fractions

From the 80% methanolic extract of stem bark of *P. linearifolia* a total of 151 g (12.6% yield) of brownish colored dry extract was harvested. While the n- hexane (0.15%) and the chloroform (2.85%) fractions were found to be greenish whereas, the dark reddish for ethyl acetate (4%) and reddish color for aqueous fraction (93%) were found. n-Hexane fraction was not considered in the experiment due to insufficient quantity.

4.2. Preliminary Phytochemical screening

Phytochemical screening of the hydroalcoholic crude extract of stem bark of *P. linearifolia* revealed the presence of alkaloids, saponins, phenolic compounds, tannins and terpenoids. Whereas, flavonoids were absent from the crude extract.

Table 1. Preliminary phytochemical screening of the crude hydroalcoholic extract of *P. linearifolia*

Phytochemical	**Result**
Alkaloids	+
Saponins	+
Flavonoids	-
Terpenoids	+
Phenols	+
Tannins	+

+ = present; - = absent

4.3. Acute oral toxicity study

The acute oral toxicity study indicated that the extract caused no mortality in 2 g/kg single dose within the first 24 hour as well as for the following 14 days. Physical and behavioral observations of the experimental mice also revealed no visible signs of toxicity like lacrimation, loss of appetite, tremors, hair erection, salivation, diarrhea and the like. This suggests that the oral LD50 of the extract is greater than 2 g/kg.

4.4. Antimalarial activity

4.4.1. Four day suppressive test of the crude extract

The results of the study showed that the crude extract of *P. linearifolia* displayed a statistically significant parasitemia level reduction dose dependently compared to vehicle treated mice ($p<0.05$ for 200 and 400 mg/kg; $p<0.001$ for 600 mg/kg) (Table 2). In addition, percentage inhibition analysis indicated that the extract produced a dose-dependent chemosuppression. Moreover, 600 mg/kg was capable of significantly increasing survival time ($p<0.05$) compared to vehicle treated mice, but the effect was significantly lower than the standard drug chloroquine 25 mg/kg ($p<0.001$).

4.4.2. Four day suppressive test of the solvent fractions

All the three doses of aqueous fraction dose-dependently reduced parasitemia level compared to vehicle treated group ($p<0.01$ for 100; $p<0.001$ for 200 and 400 mg/kg), although the extent of reduction was less than that of chloroquine 25 mg/kg, which produced 100% suppression (Table 2). The two higher doses of the chloroform fraction (200 and 400 mg/kg) dose-dependently reduced parasitemia level compared to vehicle treated group ($p< 0.01$ for 200 mg/kg; $p<0.001$ for 400 mg/kg). However, only 400 mg/kg of ethyl acetate fraction reduced parasitemia level significantly ($p<0.05$). The rank order of chemosuppression of the solvent fractions was aqueous (53.4%) > chloroform (41.11%) > ethyl acetate (35.51%), at the dose of 400 mg/kg. Survival date was significantly prolonged by 400 mg/kg of all the fractions as compared to vehicle treated mice ($p<0.05$) (Table 2).

Table 2. Parasitemia level and survival time of infected mice treated with crude extract and solvent fractions of *P. linearifolia* in the 4 day suppressive test

Extract/Fraction	**Dose**	**Parasitaemia level**	**% suppression**	**Survival time**
Crude extract of	200 mg/kg	24.57±3.29^{a1,b3}	38.17±8.28	8.60±0.67
P. linearifolia	400 mg/kg	22.52±3.40^{a1,b2}	43.33±8.56	11.60±2.56
	600 mg/kg	17.10±2.88^{a3,b1}	56.98±7.25	16.4±4.20$^{a1,b\ 2}$
Distilled water	10 ml/kg	39.74±4.99	-	6.80±0.37
Aqueous fraction	100 mg/kg	25.29±3.23^{a2,b3}	39.06±7.78	8.40±0.5^{b3}
	200 mg/kg	22.69±3.22^{a3,b3}	45.37±7.75	9.80±1.06^{b3}
	400 mg/kg	19.34±2.13^{a3,b3}	53.40±5.14	15.80±3.92^{a1,b3}
Ethyl acetate fraction	100 mg/kg	34.87±3.94^{b3}	15.99±9.49	8.40±0.74$^{b\ 3}$
	200 mg/kg	33.72±4.40^{b3}	20.70±9.40	10.40±0.92^{b3}
	400 mg/kg	26.77±2.29^{a1,b3}	35.51±5.53	10.80±1.39$^{a1,\ b\ 3}$
Chloroform fraction	100 mg/kg	31.24±2.57^{b3}	24.74±6.20	8.40±1.14^{b3}
	200 mg/kg	26.98±3.01^{a2,b3}	35.00±7.26	10.00±1.58$^{b\ 3}$
	400 mg/kg	24.44±2.89^{a3,b3}	41.11±6.96	14.00±5.00^{a2,b3,c1}
2% Tween 80	10 ml/kg	41.52±2.81	-	7.00±0.31
Chloroquine	25 mg/kg	0.00±0.00^{a3}	100.00±0.00	30.00±0.00^{a3}

Data are expressed as mean ± SEM; n = 5; a, compared to vehicle (Distilled water for crude extract and 2%Tween 80 10ml/kg for fractions); b to chloroquine 25 mg/kg; c to chloroform fraction 100 mg/kg: 1p<0.05, 2p<0.01, 3p<0.001.

4.4.3. Effect of crude extract and solvent fractions on rectal temperature and body weight on the four day suppressive test

Analysis of the rectal temperature revealed that 600 mg/kg of crude extract caused a statistically significant attenuation of reduction in temperature of *P. berghei* infected mice ($p<0.05$), but it was lower than chloroquine 25 mg/kg ($p<0.001$). All doses of the crude extract failed to prevent the reduction in body weight significantly; but the standard drug chloroquine 25 mg/kg averted it significantly ($p<0.001$) (Table 3).

Analysis of rectal temperature within groups showed that groups of mice treated with 200 mg/kg of the crude extract did not show any significant temperature change after treatment. Similarly the standard drug chloroquine 25 mg/kg prevented significant temperature reduction. But other doses did not show any significant prevention of the reduction in rectal temperature. Also analysis of body weight indicated that mice administered with the standard drug chloroquine 25 mg/kg did not show any significant weight change after treatment. All doses of the crude extract failed to prevent a significant weight change after treatment.

All doses of the fractions, except 400 mg/kg chloroform extract failed to prevent body weight reduction significantly. But, 400 mg/kg of chloroform showed a significant attenuation of body weight reduction ($p<0.05$). On the other hand, the standard drug chloroquine 25 mg/kg significantly prevented body weight reduction ($p<0.01$) (Table 3). Moreover, all doses of the aqueous fraction significantly ($p<0.001$) prevented the rectal temperature reduction caused by escalating parasitemia and the effect was comparable to that observed with chloroquine 25 mg/kg. Similarly, all doses of chloroform fraction ($p<0.01$), 200 and 400 mg/kg of ethyl acetate fraction ($p<0.05$) significantly prevented rectal temperature reduction (Table 3).

Analysis of rectal temperature change within groups indicated that mice administered with 100 mg/kg and 400 mg/kg of the aqueous fraction did not show any significant temperature change after treatment. In addition, chloroquine 25 mg/kg prevented a significant rectal temperature change. But ethyl acetate and chloroform fractions did not show any significant prevention of temperature change. On the other hand there was no significant weight change after treatment by group of mice treated with chloroquine 25 mg/kg. No dose of all the three fractions prevent weight reduction significantly.

Table 3 Rectal temperature and body weight change of infected animals treated with crude extract and solvent fractions of *P. linearifolia* in the 4 day suppressive test.

Extract/ Fraction	**Dose**	**Temperature (℃)**			**Weight**		
		D0	**D4**	**% Change**	**D0**	**D4**	**% change**
Crude extract of *P. linearifolia*	200	37.18±0.32	36.46±0.2	-1.93	28.8±1.11	26.74±0.92	-7.15
	400	37.68±0.05	37.04±0.16	-1.69	27.72±1.00	25.76±1.20	-7.07
	600	37.58±0.09	37.20±0.11	-1.01^{a1}	26.9±1.23	24.92±1.38	-7.36
D/water	10	36.76±0.22	35.16±0.29	-4.35	30.42±0.72	26.56±0.89	-12.68
Chloroquine	25	36.76±0.33	36.9±0.24	0.38^{a3}	27.52±1.70	27.38±1.42	-0.50^{a2}
Aqueous fraction	100	37.22±0.08	36.84±0.12	-1.02^{a3}	25.78±1.07	22.40±1.27	-13.11^{b2}
	200	37.52±0.23	37.18±0.18	-0.90^{a3}	27.46±1.55	24.46±1.76	-10.92^{b2}
	400	37.48±0.08	37.42±0.07	-0.16^{a3}	26.54±1.27	23.9±1.49	-9.94^{b2}
Ethyl acetate	100	37.06±0.12	35.90±0.17	-3.13	27.56±1.16	24.38±1.13	-11.53^{b2}
Fraction	200	37.42±0.10	36.62±0.24	-2.13^{a1}	25.42±0.98	23.26±0.81	-8.49^{b1}
	400	37.14±0.10	36.72±0.19	-1.13^{a2}	27.20±1.06	24.80±1.39	-8.82^{b1}
Chloroform	100	37.3±0.16	36.70±0.15	-1.60^{a2}	28.06±1.41	24.92±1.49	-11.19^{b3}
Fraction	200	37.82±0.09	37.06±0.13	-2.02^{a2}	27.66±1.20	25.00±1.14	-9.59^{b2}
	400	37.5±0.07	37.06±0.12	-1.17^{a2}	27.72±1.24	25.74±1.16	-7.14^{a1,b1}
2%Tween80	10	37.76±0.23	36.12±0.30	-4.34	29.38±0.91	25.04±0.99	-14.77
Chloroquine	25	37.08±0.22	36.94±0.16	-0.37^{a3}	26.66±1.34	26.56±1.34	-0.37^{a2}

Data are expressed as mean ± SEM; n = 5; a = compared to vehicle (Distilled water for crude extract and 2%Tween 80 10ml/kg for fractions); b to CQ: 1p< 0.05; 2p<0.01, 3p<0.001; D0 = pre-treatment value on day 0, D4 = post-treatment value on day four, Numbers refer to dose in mg/kg.

4.4.4. Effect of crude extract and solvent fractions on packed cell volume (PCV) on the four day suppressive test

Both 400 and 600 mg/kg of the crude extract significantly prevented the reduction ($p<0.05$ for 400 mg/kg and $p<0.01$ for 600 mg/kg) as compared to vehicle treated mice. However, the effect was lower than chloroquine 25 mg/kg ($p<0.001$). Moreover, within group analysis of PCV indicated that neither the standard drug nor the crude extract prevented any significant PCV change after treatment (Figure 4).

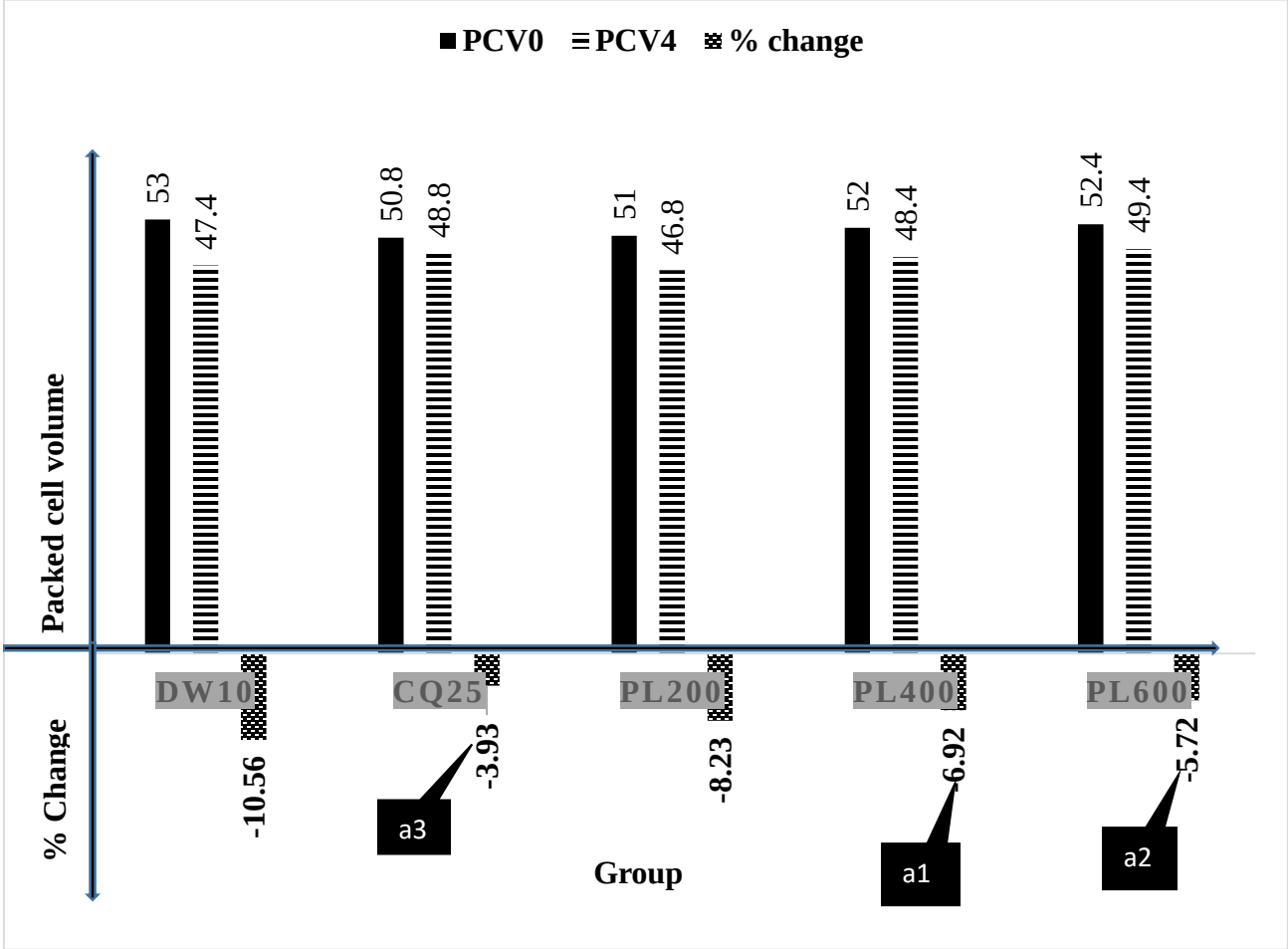

Figure 4. Packed cell volume of infected animals treated with crude extract of *P. linearifolia* in the 4 day suppressive test. Data are expressed as mean ± SEM; n = 5; a = compared to Vehicle (Distilled water, 10ml/kg): 1p< 0.05; 2p<0.01, 3p<0.001; PCV0 = pre-treatment value on day 0, PCV4 = post-treatment value on day four, CQ = chloroquine, PL, crude extract of *P. linearifolia.* Numbers refer to dose in mg/kg.

PCV reduction was significantly attenuated by 400 mg/kg chloroform fraction ($p<0.01$). Also 200 and 400g mg/kg of the aqueous fraction showed a significant effect in preventing PCV ($p<0.05$ for both doses); but the effect was lower than the standard drug chloroquine 25 mg/kg ($p<0.001$). On the other hand, 100 and 200 mg/kg of the chloroform fraction, 100 mg/kg aqueous fraction and all doses of the ethyl acetate fraction failed to prevent the reduction in packed cell volume significantly (Figure 5). Similarly, within group analysis indicated all fractions failed to prevent PCV reduction after treatment except chloroquine 25 mg/kg which prevented significant PCV change after treatment.

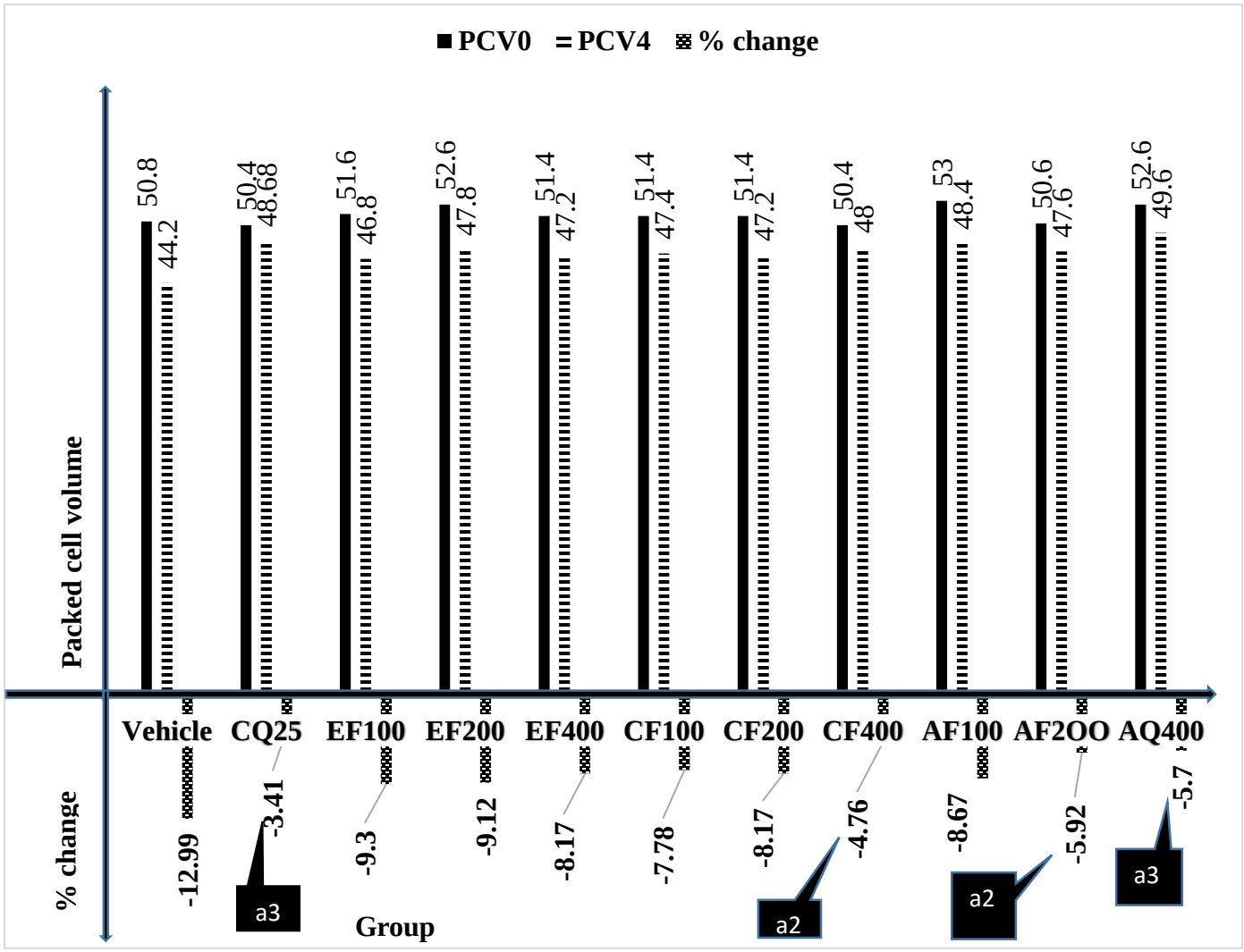

Figure 5. Packed cell volume of infected animals treated with solvent fractions of *P. linearifolia* in the 4 day suppressive test. Data are expressed as mean ± SEM; n = 5; a = compared to vehicle (2% Tween 80 10 ml/kg): 1p< 0.05; 2p<0.01, 3p<0.001; PCV0 = pre-treatment value on day 0, PCV4 = post-treatment value on day four, CF = Chloroform fraction, CQ = chloroquine, EF, Ethyl acetate fraction, AF, aqueous fraction. Numbers refer to dose in mg/kg.

4.4.5. Rane's test of the crude extract

On this antimalarial activity test on established (late) infection only 600 mg/kg of the crude extract of *P. linearifolia* revealed a significant parasite level reduction ($p<0.01$) compared to vehicle treated mice (Table 4). The standard drug chloroquine 25 mg/kg showed a highly significant parasite level reduction ($p<0.001$) which cured all mice at day seven. Both 200 and 400 mg/kg of the crude extract failed to reduce parasitemia level significantly as compared to vehicle treated mice. Survival time was not altered by all doses of the crude extract. However, the standard drug chloroquine 25 mg/kg significantly prolonged survival time of the mice as compared to vehicle treated mice ($p<0.001$). Between different doses comparison analysis showed that 600 mg/kg prevented parasitemia level significantly higher than 400 mg/kg (Table 4).

4.5.6. Rane's test of the aqueous fraction

The fraction with the highest antimalarial activity (aqueous fraction) in the four day suppressive test was further evaluated for its effect on established parasite infection. In this case only 400 mg/kg of the aqueous fraction exhibited a statistically significant parasite level reduction ($p<0.01$) as compared to vehicle treated mice. On the other hand, chloroquine 25 mg/kg cured all mice on day 7 and the reduction in parasitaemia was significantly higher ($p<0.001$) when compared to vehicle treated mice as well as all doses of the fraction. Only 400 mg/kg of the aqueous fraction significantly prolonged survival time of mice compared to vehicle treated mice ($p<0.05$) (Table 4). Comparison analysis between the group showed 400 mg/kg of the aqueous fraction's effect in parasitemia level reduction was significantly higher than 100 and 200 mg/kg ($p<0.05$) (Table 4).

Table 4. Parasitaemia and survival time of infected animals treated with crude extract and aqueous fraction of *P. linearifolia* in the Rane's test

Dose	Parasitemia level					%Inhibition	Survival Time
	Day3	Day4	Day5	Day6	Day7		
PL200	10.08±2.06	28.74±5.56	49.36±5.90	53.99±5.74	61.25±8.87^{b3}	22.22±11.26	8.40±0.50^{b3}
PL400	11.01±1.80	34.01±3.90	47.60±2.01	56.47±3.52	64.28±2.66^{b3}	18.37±3.38	8.80±0.66^{b3}
PL600	8.64±1.41	25.02±4.43	36.01±4.22	38.75±3.58	41.89±2.77^{a3,b3,c1}	46.79±3.52	10.40±1.36^{b3}
AF100	11.26±1.62	25.30±4.16	39.66±3.78	53.36±2.60	69.93±2.70^{b3}	11.18±3.42	9.00±0.70^{b3}
AF200	9.01±1.49	26.28±3.87	40.42±2.47	55.12±2.39	69.84±2.05^{b3}	11.30±2.60	9.60±0.81^{b3}
AF400	8.87±1.92	12.47±3.24	21.92±6.79	27.14±9.16	39.86±11.62^{a2,b2,d1,e1}	49.37±14.76	12.00±1.51^{a1,b3}
CON.	11.52±1.86	37.39±5.74	61.62±6.87	66.96±5.70	78.75±5.60	-	7.60±0.24
CQ25	9.56±3.04	9.41±3.71	1.10±0.40	0.32±0.23	0.00±0.00^{a3}	100.00±0.00	30.00±0.00 ^{a3}

Data are expressed as mean ± SEM; n = 5: a, compared to negative control (Distilled water); b to CQ; c to PL400; d to AF100; e to AF200: 1p<0.05, 2p<0.01, 3p<0.001; AF = Aqueous fraction, CQ = chloroquine, EF, ethyl acetate fraction, AF, aqueous fraction, CON = Negative control, PL, crude extract of *P. linearifolia.* Numbers refer to dose in mg/kg.

4.4.7. Effect of crude extract and aqueous fraction on the body weight and rectal temperature in the Rane's test

Rectal temperature analysis indicated that 400 and 600 mg/kg of *P. linearifolia* had a significant preventive effect on the reduction in rectal temperature compared to vehicle treated mice (p<0.05). The effect of chloroquine 25 mg/kg on prevention of rectal temperature reduction was much higher than the extract (p<0.001) (Table 5). Weight reduction was not significantly prevented by all doses of the crude extract. However, chloroquine 25 mg/kg significantly averted weight reduction as compared to vehicle treated mice (p<0.01) (Table 5).

Analysis of rectal temperature change of mice within groups showed that all doses of the crude extract failed to prevent any significant rectal temperature change after treatment. But, the standard drug chloroquine 25 mg/kg averted a significant temperature change after treatment. All doses of the crude extract failed to prevent weight change after treatment except the standard drug chloroquine 25 mg/kg.

On the other hand, only 400 mg/kg of the fraction significantly prevented the reduction in the temperature compared to vehicle treated mice (p<0.05). However, its effect was much lower than chloroquine 25 mg/kg (p<0.001). Chloroquine 25 mg/kg halted temperature dropping significantly compared to all doses of the

fraction ($p<0.05$). Only 400 mg/kg of the aqueous fraction prevented body weight loss significantly as compared to vehicle treated mice ($p<0.05$). The other doses of the fraction failed to attenuate body weight reduction significantly (Table 5).

Analysis of rectal temperature change of mice within groups showed that all doses of the aqueous fraction failed to prevent a significant rectal temperature change after treatment. Only the standard drug chloroquine 25 mg/kg averted significant rectal temperature reduction. Also, mice administered with 400 mg/kg of the aqueous fraction did not show any significant weight change after treatment.

Table 5. Body weight and rectal temperature change of infected mice treated with crude extract and solvent fraction of *P. linearifolia* in the Rane's test

	Weight			**Temperature**		
Dose	**W0**	**W4**	**% Change**	**T0**	**T4**	**% Change**
PL200	27.90±1.08	24.74±0.80	-11.32^{b2}	36.98±0.17	34±0.46	-7.67^{b2}
PL400	27.08±1.00	23.68±0.89	-12.55^{b2}	36.64±0.12	34.32±0.35	-6.33^{a1,b1}
PL600	26.92±1.29	24.62±0.79	-8.54^{b1}	37.12±0.11	34.52±0.43	-7.00^{a1,b2}
AF100	27.98±0.95	26.32±1.10	-5.93	37.62±0.13	33.40±0.14	-11.21^{b3}
AF200	28.16±1.84	26.38±1.60	-6.32	37.18±0.26	34.20±0.35	-8.01^{b2}
AF400	29.12±0.99	28.24±1.36	-3.02^{a1}	37.86±0.14	35.94±0.47	-5.07 ^{a1,c2}
CON.	27.26±1.13	23.64±1.27	-13.27	36.74±0.21	32.44±0.20	-11.70
CQ25	26.08±1.20	26.56±1.39	1.9^{a2}	36.64±0.17	36.22±0.24	-1.14^{a3}

Data are expressed as mean ± SEM; n = 5: a, compared to control; b to CQ; c to AF100: 1p<0.05, 2p<0.01, 3p<0.001; AF = Aqueous fraction, CQ = chloroquine, EF, ethyl acetate fraction, AF, aqueous fraction, CON = Control, PL, crude extract of *P. linearifolia.* Numbers refer to dose in mg/kg.

4.4.8. Effect of crude extract and aqueous fraction on packed cell volume in the Rane's test

Only 600 mg/kg of the crude extract had significantly prevented PCV reduction as compared to vehicle treated mice ($p<0.05$) (Figure 6). Within group analysis showed both chloroquine 25 mg/kg and all doses of the crude extract were unable to prevent any significant PCV change after treatment.

All doses of the aqueous fraction failed to prevent PCV reduction significantly. However, the standard drug chloroquine 25 mg/kg averted PCV reduction significantly as compared to vehicle treated mice ($p<0.01$). On the other hand, within group analysis indicated that neither the fraction nor the standard drug prevented a significant PCV change after treatment (Figure 6).

Figure 6. Packed cell volume of infected animals treated with crude extract and solvent fraction of *P. linearifolia* in the Rane's test

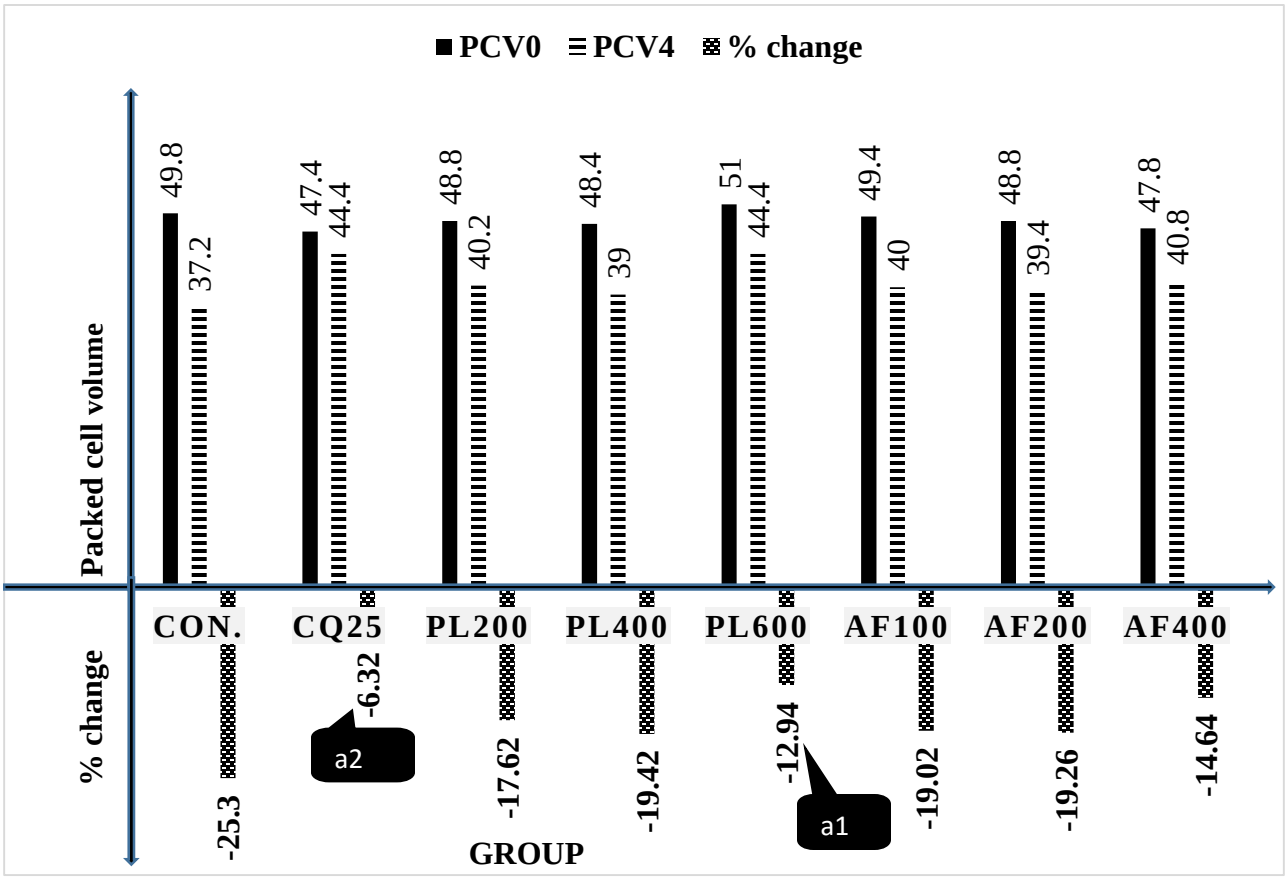

Figure 6. Packed cell volume of infected animals treated with crude extract and solvent fraction of *P. linearifolia* in the Rane's test. Data are expressed as mean ± SEM; n = 5: a, compared to control; b, to 600 mg/kg; c, to CQ 25; d, to 400 mg/kg; e, to 200 mg/kg: 1p<0.05, 2p<0.01, 3p<0.001; AF = Aqueous fraction, CQ = chloroquine, EF, ethyl acetate fraction, AF, aqueous fraction, CON = Control, PL, crude extract of *P. linearifolia*. Numbers refer to dose in mg/kg.

5. DISCUSSION

The *in vivo* model was preferred for this study because it takes into account the possible prodrug effect and possible involvement of the immune system in eradication of infection (103). *P. berghei* provides a well-established experimental model of malaria infection (104), producing pathological symptoms which closely mimic those of human malaria (105). Even though the rodent malaria model is not exactly similar to that of the human *Plasmodium* parasites, it is the first step to screen most of the *in vivo* antimalarial activities of test compounds (97, 101).

In addition, several of the currently available antimalarial agents such as chloroquine, halofantrine, mefloquine and artemisinin derivatives have been identified using rodent model of malaria (106). The 4 day suppressive test, which mainly evaluates the antimalarial activity of candidates on early infections, and Rane's test, which evaluates the curative capability of candidate extracts on established infections, are commonly used for antimalarial drug screening. In both methods, determination of percent inhibition of parasitemia is the most reliable parameter (97, 101, 102).

It can be clearly seen from the results that percentage parasitemia measured in the 4 day suppressive test was reduced dose dependently by the crude extract in *P. berghei* infected mice, pointing to the fact that the plant is endowed with antimalarial activity. Alkaloids and terpenoids present in the extract might be responsible for their antimalarial activity. The alkaloids are known to possess antiplasmodial properties, the most famous being quinine (107). Survival time was significantly prolonged by the crude extract and aqueous fraction which is related with reduction of parasitemia level.

Among the fractions, the aqueous fraction was found to possess higher antimalarial activity than the chloroform and ethyl acetate fractions. Hence in this study the hydroalcoholic extract and aqueous fraction showed higher antiplasmodial activity than the chloroform and ethyl acetate fractions. The observed activities of the hydrophilic extracts are in contrast to those reported by Jenett Siems *et al*., who found that lipophilic extracts were more active than hydrophilic extracts (108). Hydrophilic extracts are closer in composition to the aqueous preparations commonly used by traditional practitioners. Francois *et al*. have similarly investigated the organic extracts from the roots, stem bark, fruit rind, seeds and leaves of plants and found a wide range of activities with the highest activities being found in the methylene chloride root extracts (109). Methanolic and chloroform extract of *P. linearifolia* is reported to possess *in vitro* antiplasmodial activity (92). Therefore, the present *in vivo* study is consistent with the previous *in vitro* findings.

Different ethnobotanical studies indicated that from the sub family Periplocoideae (Apocynaceae), where *P. linearifolia* belonged, many plant species are used for the treatment of malaria. Similarly, phytochemical

analysis studies showed these plants contain different alkaloids and terpenoids which had a promising antiplasmodial activities in *in vitro* studies (80-85). Moreover, many chemical compounds have been isolated and identified from species of the *Periploca* genus, such as *α*- and *β*-amyrin, lupeol and *β*-sitosterol from *Periploca laevigata* (86). Moreover, several lupene-type triterpenes and elemane-type sesquiterpenes have been isolated from *Periploca aphylla* and roots of *P. laevigata* respectively (86, 87). Lupeol, *β*-amyrin and *β*-sitosterol (terpenoids), inhibited both chloroquine sensitive and resistant strains of *P. falciparum* in *in vitro* studies (88). Therefore, the antiplasmodial activity of *P. linearifolia* in the present *in vivo* study is in agreement with other plants belonging to the same sub family and genus.

The methanol extracts of the root bark of *P. linearifolia* showed anti-oxidant activity dependent on concentration. Three compounds namely, Lupeol, β- sitosterol and β- amyrin were isolated from the root bark of *P. linearifolia.* The three compounds belonged to the terpenoid class of compounds inhibited both chloroquine sensitive and chloroquine resistant strains of *P. falciparum* (88). Similarly, the present *in vivo* study is consistent with the above finding.

Anemia, body weight loss and body temperature reduction are the general features of malaria infected mice (110). PCV was measured to evaluate the effectiveness of the crude extract and fractions in preventing hemolysis due to escalating parasitemia level. The underlying cause of anemia includes the following mechanisms; the clearance and/or destruction of infected RBCs, the clearance of uninfected RBCs, and erythropoietic suppression and dyserythropoiesis. Each of these mechanisms has been implicated in both human and mouse malarial anemia (111). This necessitates hematocrit (packed cell volume) analysis that evaluates the effectiveness of the extract in preventing hemolysis. In the four day suppressive test 200 mg/kg of the aqueous extract, 400 mg/kg of the crude extract, chloroform and aqueous fractions and 600 mg/kg of the crude extract prevented PCV reduction significantly. In addition, in the Rane's test 600 mg/kg was able to avert significant PCV reduction. This reversal of PCV reduction by the crude extract and fractions may be related with parasitemia level reduction with the extract and fractions.

The crude extract and most doses of the fractions failed to prevent significantly the reduction in body weight. This phenomenon in decrement of body weight may be due to disturbed metabolic function and hypoglycaemia that has been reported to be associated with malaria infection (112).

Rectal temperature measurement showed that the mice developed hypothermia particularly in the late infection (Rane's test) with *P. berghei* parasites. Fever is one of the symptoms of human malaria. In contrast, this model of malaria was associated with hypothermia rather than pyrexia. In the curative test, the infected mice developed profound hypothermia with rectal temperature falling by as much as 5°C. The prolonged

development of hypothermia in mice can be attributed to the general debilitating effects of malaria on the host, which results in the loss of body heat and eventual death. The absence of pyrogenic response in this model is that small animals like mice have a large surface area-to-body mass ratio, which resulted in a higher degree of heat loss and prevents the development of fever caused by pyrogenic agents (113).

Malaria parasites also affect host carbohydrate, lipid and protein metabolism (114, 115). A decrease in the metabolic rate of infected mice occurred just before death and was accompanied by a corresponding decrease in internal body temperate (116). Therefore, effective antimalarial agents are expected to prevent the reduction in rectal temperature. As a result, the crude extract and fractions were able to significantly attenuate the reduction in rectal temperature as compared to vehicle treated mice.

In the curative test, blood samples were taken and smears prepared daily to evaluate the curative ability of the extract. As indicated in the results section, 600 mg/kg of the crude extract brought about reduction of parasitemia after second dose. However, 400 mg/kg of the aqueous fraction and the standard drug chloroquine 25 mg/kg started parasitemia reduction activity right after the first dose. This delay of activity of 600 mg/kg of the crude extract may be indicative of the need for a loading dose or the extract might have a delayed onset of action. Generally, the results obtained from the Rane's test suggest that 600 mg/kg of the crude extract and 400 mg/kg of the aqueous fraction have therapeutic efficacy against established malaria parasite. This property is additive to the suppressive activity and it may be possible to consider the plant as a potential source of antimalarial agents (117).

Although the active compound is yet to be identified, the antimalarial activity of *P. linearifolia* could be attributed to a single or a combination of its secondary metabolites such as alkaloids, terpenoids and phenolic compounds. These metabolites have been reported to have different extent of antimalarial activity in the literature (118-120).

Anti-malarial test materials with more than 30% suppressive effect on the level of parasitaemia (121, 122) or that can prolong the survival date of treated mice (123) compared to the control group are often considered effective in standard screening tests. Therefore, the present *in vivo* antimalarial activity test denotes *P. linearifolia* is effective in both the four day suppressive test and curative test models.

6. CONCLUSION

From the results of this study it can be concluded that the plant extract is relatively safe to mice. The present study indicates that 80% methanolic extract and solvent fractions of *P. linearifolia* have a promising antiplasmodial activity, with varying degree and/or differential effect on the measured parameters. The crude extract appeared to be superior in suppressing parasitemia and where 400 mg/kg and 600 mg/kg protected infected mice from parasite-induced PCV reduction. Moreover, 400 mg/kg of the chloroform fraction and 200 mg/kg and 400 mg/kg of the aqueous fraction protected PCV reduction and displayed greater parasite suppression among the fractions. The results reported in this study illustrate that correlations exist between the traditional use and the earlier *in vitro* findings.

7. RECOMMENDATION

From the present study, the following works are suggested for further investigation on the plant:

- Active compounds, responsible for the antimalarial activity, have to be isolated
- Mechanism of antimalarial action of the compounds has to be studied in detail.
- Sub-acute and chronic toxicity study in higher animals has to be conducted to know the safety of *P. linearifolia* stem bark.

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